Anti-Mycotics Suppress Interleukin-4 and Interleukin-5 Production in Anti-CD3 Plus Anti-CD28-Stimulated T Cells from Patients with Atopic Dermatitis

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It is reported that anti-mycotic agents are effective for the treatment of patients with atopic dermatitis. We studied the *in vitro* effects of anti-mycotics on T helper-1 and T helper-2 cytokine production in anti-CD3 plus anti-CD28-stimulated T cells from atopic dermatitis patients and normal donors. The amounts of interleukin-4 and interleukin-5 secreted by anti-CD3/CD28-stimulated T cells were higher in atopic dermatitis patients than in normal donors. Azole derivatives, ketoconazole, itraconazole, miconazole, and nonazole terbinafine hydrochloride, and tolnaftate reduced interleukin-4 and interleukin-5 secretion without altering that of interferon-γ and interleukin-2 in anti-CD3/CD28-stimulated T cells from both atopic dermatitis patients and normal donors. The azole derivatives were more inhibitory than nonazole anti-mycotics. These anti-mycotics reduced the anti-CD3/CD28-induced mRNA expression and promoter activities for interleukin-4 and interleukin-5. The 3′,5′-cyclic adenosine monophosphate analog dibutyryl 3′,5′-cyclic adenosine monophosphate reversed the inhibitory effects of the anti-mycotics on interleukin-4 and interleukin-5 secretion, mRNA expression, and promoter activities. Anti-CD3/CD28 transiently (⩽ 5 min) increased intracellular 3′,5′-cyclic adenosine monophosphate in T cells, and the increase was greater in atopic dermatitis patients than in normal donors. The increase of 3′,5′-cyclic adenosine monophosphate by anti-CD3/CD28 correlated with interleukin-4 and interleukin-5 secretion by anti-CD3/CD28. The transient 3′,5′-cyclic adenosine monophosphate increase was suppressed by anti-mycotics, and azole derivatives were more suppressive than nonazoles. Azole derivatives inhibited the activity of cyclic adenosine monophosphate-synthesizing adenylate cyclase whereas terbinafine hydrochloride and tolnaftate enhanced the activity of 3′,5′-cyclic adenosine monophosphate-hydrolyzing cyclic nucleotide phosphodiesterase in atopic dermatitis and normal T cells. These results suggest that the anti-mycotics may suppress interleukin-4 and interleukin-5 production by reducing 3′,5′-cyclic adenosine monophosphate signal, and stress their potential use for the suppression of T helper-2-mediated allergic reactions. *Key words: adenylate cyclase/cyclic adenosine monophosphate/cyclic nucleotide phosphodiesterase J Invest Dermatol 117:1635–1646, 2001*

It has recently been reported that systemic and/or topical treatment with anti-mycotics, especially azole derivatives such as ketoconazole, is effective for patients with atopic dermatitis (AD) (Clemmensen and Hjorth, 1983; Back *et al*, 1995; Broberg and Faergemann, 1995). This is partly caused by the fungicidal or fungistatic effects of these agents, such as the inhibition of ergosterol synthesis, cell membrane damage, disruption of microtubule function, or accumulation of squalene (Elewski, 1993; Agut *et al*, 1992), as skin-localized fungi such as *Malassezia furfur* may act as allergens for IgE-mediated allergic responses in AD (Tengvall Linder *et al*, 1996). Additional mechanisms unrelated to fungi, however, may also exist in the therapeutic efficacy of anti-mycotics on AD; ketoconazole inhibits the synthesis of leukotrienes via inhibiting 5-lipoxygenase in polymorphonuclear leukocytes (Bettens *et al*, 1986); also, this effect may contribute to the attenuation of AD symptoms as the synthesis of leukotriene B4, which induces cutaneous inflammation, is enhanced in AD skin lesions (Fogh *et al*, 1989). Ketoconazole also inhibits lipopolysaccharide-induced expression of the nitric oxide synthase gene and consequently inhibits reactive radical nitric oxide production in macrophages, which may explain the anti-erythema and anti-edema action of ketoconazole (Baroni *et al*, 1999). Other immunomodulatory effects of anti-mycotics have also been reported; ketoconazole inhibited the interleukin (IL)-2-dependent proliferation of human T cell clones or proliferation of human peripheral blood mononuclear cells in allogeneic mixed lympho-

Manuscript received May 30, 2001; revised July 31, 2001; accepted for publication August 4, 2001.

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Abbreviations: AC, adenylate cyclase; AD, atopic dermatitis; Bt_2, dibutyryl; cAMP, 3′,5′-adenosine cyclic monophosphate; CAT, chloramphenicol acetyl transferase; C/EBP, CCAAT/enhancer-binding protein; cGMP, 3′,5′-guanosine cyclic monophosphate; CREB, cAMP response element binding protein; DMEM/F-12, 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's Nutrient Mixture F-12; Gi, inhibitory guanine-nucleotide-binding protein; Gs, stimulatory guanine-nucleotide-binding protein; H-89, N-[2-((p-bromocinnamyl)aminoethyl)-5-isoquinolinesulfonamide; OAG, 1-oleoyl-2-acetyl-*sn*-glycerol; PDE, cyclic nucleotide phosphodiesterase; PKA, protein kinase A; Th1, T helper 1.

cyte culture (Pawelec *et al*, 1991a). Another azole derivative itraconazole also inhibited the generation of allospecific cytolytic activity in human mixed lymphocyte culture (Pawelec *et al*, 1991b). It is thus anticipated that anti-mycotics may modulate certain aspects of allergic immune responses in AD and thus alleviate the symptoms of AD.

It has been proposed that T helper (Th)-2-type cytokines, such as IL-4, IL-5, IL-10, or IL-13, play key pathogenetic roles in AD (Grewe *et al*, 1998); T cells from AD patients predominantly produce Th2 type cytokines in response to inhalant allergens such as house dust mite (Wierenga *et al*, 1991). In particular, IL-4 enhances IgE synthesis and IL-5 favors the differentiation of eosinophils; the overexpression of these two cytokines may induce the increased serum IgE levels and blood eosinophilia in the majority of AD patients (Grewe *et al*, 1998). In contrast, previous studies reported that the production of Th1 type cytokines, such as interferon (IFN)-γ, by T cells in AD patients was reduced compared with that in nonatopic donors (Reinhold *et al*, 1990; Rousset *et al*, 1991; Katagiri *et al*, 1997).

The recent successful results in the treatment of AD patients with anti-mycotics indicate that these agents may manipulate the production of Th1 and/or Th2 cytokines in AD patients. It is reported that treatment with ketoconazole reduced the serum IgE level in AD patients (Back *et al*, 1995), indicating that ketoconazole may suppress the production of Th2 cytokines promoting IgE synthesis, such as IL-4 or IL-13, and/or upregulate the production of Th1 cytokines inhibiting IgE synthesis, such as IFN-γ. The treatment with anti-mycotics amphotericin B or fluconazole decreased IL-4 production and increased that of IFN-γ in *Candida albicans*-infected mice, alone or in combination with anti-IL-10, soluble IL-4 receptor, or recombinant IL-12 (Cenci *et al*, 1997; Mencacci *et al*, 2000). The treatment with new azole anti-mycotics SCH 42427 or SCH 39304 also reduced serum IgE level in *Paracoccidioides brasiliensis* or *Blastomyces dermatidis*-infected mice, respectively, suggesting the suppression of Th2 responses by these agents (Brummer *et al*, 1993; Hostetler *et al*, 1993).

In this study, we examined the *in vitro* effects of anti-mycotics on the production of Th2 cytokines, IL-4 and IL-5, and Th1 cytokines, IFN-γ and IL-2 in anti-CD3 plus anti-CD28-stimulated T cells from AD patients, and the effects were compared with those in nonatopic donors. The anti-mycotics examined were azole derivatives, ketoconazole, itraconazole, miconazole, and nonazole anti-mycotics, terbinafine hydrochloride and tolnaftate, which are often used topically or systemically for the treatment of AD. We have obtained the results that these agents reduced Th2 cytokine production without altering that of Th1. We further analyzed the mechanism for the inhibitory effects focusing on the 3′,5′-adenosine cyclic monophosphate (cAMP)-mediated signaling pathways in T cells.

MATERIALS AND METHODS

Patients and controls We studied 15 patients with mild to severe AD [eight men and seven women, age 25.7 ± 2.4 y (mean ± SEM)], diagnosed according to the criteria by Hanifin and Rajka (1980). Their disease severity was scored according to the grading by Rajka and Langeland (1989), and the score was 5.2 ± 0.6 (mean ± SEM). The patients' serum total IgE value was 4658 ± 1707 U per ml (mean ± SEM). At the time of the study, 12 of 15 patients were treated with topical corticosteroids of mild to very strong rank. No patients were taking systemic corticosteroids, anti-allergic or antihistaminic drugs, or receiving desensitization immunotherapy. Fourteen nonatopic healthy volunteers [seven men and seven women, age 25.9 ± 2.1 y (mean ± SEM)] were studied as controls. None of the controls had symptoms indicative of allergic diseases, or receiving medication, and their serum total IgE value was 74 ± 11 U per ml. At the time of the investigation, all the patients and controls did not have active infection by fungi, viruses, or bacteria, and were not receiving topical or systemic anti-mycotic medication, and consumed no beverage containing caffeine or methylxanthine for at least 8 h before drawing blood. All the patients and controls were informed of the objectives and methods of this study, and consented to participate.

Reagents Anti-CD3 monoclonal antibody (OKT3, murine IgG2a) and anti-CD28 monoclonal antibody (clone 9.3, murine IgG2a) were purchased from Becton Dickinson (San Jose, CA). Dibutyryl cAMP (Bt_2cAMP), dibutyryl 3′,5′-cyclic guanosine monophosphate (Bt_2cGMP), forskolin, N-[2-((*p*-bromocinnamyl)aminoethyl)-5-isoquinolinesulfonamide (H-89), 1-oleoyl-2-acetyl-*sn*-glycerol (OAG), and 3-isobutyl-1-methylxanthine were obtained from Calbiochem (La Jolla, CA). Ketoconazole and itraconazole were from Janssen Pharmaceutica NV (Beerse, Belgium). Miconazole was provided by Mochida Pharmaceutical (Tokyo, Japan). Terbinafine hydrochloride was from Novartis Pharma (Tokyo, Japan). Tolnaftate was from Yamanouci Pharmaceutical (Tokyo, Japan). These agents were dissolved in dimethylsulfoxide as 10 mM stock solution and were kept in the dark until used.

Human T cells and T cell line Peripheral blood mononuclear cells from AD patients and normal donors were isolated by centrifugation over Ficoll-Paque (Pharmacia, Uppsala, Sweden) as described (Boyum, 1968), and were allowed to adhere to plastic dishes for 1 h at 37°C. From the nonadherent cells, $CD56^-$ cells were isolated by negative selection using immunomagnetic beads (Dynal, Great Neck, NY) as described (Gee *et al*, 1987), and were incubated with neuraminidase-treated sheep erythrocytes as described (Farrant *et al*, 1985). From the rosette-forming cells, $CD14^-$ and $CD19^-$ cells were isolated by the immunomagnetic negative selection, and were used as T cells. This T cell population was > 98% $CD3^+$, and the contamination of $CD14^+$, $CD19^+$, or $CD56^+$ cells was < 2%.

Human Jurkat T cells were purchased from Dainippon Pharmaceutical (Osaka, Japan), and were maintained in RPMI 1640 (Gibco/BRL, Grand Island, NY) supplemented with 10% fetal bovine serum, 100 U penicillin per ml, and 100 μg streptomycin per ml.

Measurement of cytokine secretion T cells (2×10^6 cells per ml) were preincubated with or without various anti-mycotics at indicated concentrations for 30 min, and seeded at the density of 4×10^5 per well in triplicate to anti-CD3 plus anti-CD28-precoated plates as described (Li *et al*, 1999a); the plates were coated by 100 μl of 10 μg anti-CD3 per ml plus 1 μg anti-CD28 per ml at 37°C for 1 h. The cells were incubated for another 48 h in 200 μl per well of culture medium at 37°C in the presence or absence of respective anti-mycotics in an atmosphere of 5% CO_2. We used endotoxin-, hormone-, and serum-free medium, 1:1 mixture of Dulbecco's minimal Eagle's medium (DMEM) and Ham's Nutrient Mixture F-12 (DMEM/F-12) (Sigma, St. Louis, USA), supplemented with 2.5 mM L-glutamine (Gibco/BRL). The activity of IL-2, IFN-γ, IL-4, and IL-5 in the culture supernatants was measured by enzyme-linked immunosorbent assay (ELISA) kits (Biosource, Tokyo, Japan) according to the manufacturer's instructions. The sensitivity of the assay for IL-2, IFN-γ, IL-4, and IL-5 was 5, 4, 3, and 4 pg per ml, respectively.

Reverse transcription–polymerase chain reaction (reverse transcription–PCR) T cells were incubated under the conditions indicated, and total cellular RNA was extracted using mRNA purification kit (Pharmacia) according to the manufacturer's instructions. cDNA was made from RNA samples as described (Llorente *et al*, 1994). Primer sequences for cytokines and for the internal control β-actin as well as PCR conditions are as described (Kanda and Watanabe, 2001). The PCR products were analyzed by electrophoresis on 2.5% agarose gels and stained with ethidium bromide. The intensity of the reverse transcription–PCR products for cytokines and β-actin was determined by densitometry (Hoefer Scientific Instruments, San Francisco, CA). Results are expressed for each cytokine product as the ratio relative to β-actin product.

Measurement of cAMP amount T cells were cultured under the indicated conditions, and were harvested and lyzed with ethanol. The cell lysates were centrifuged and the supernatants were dried under vacuum. The dried samples were dissolved in acetate buffer (pH 5.8), and cellular cAMP contents were measured with an ELISA kit from Amersham (Arlington Heights, IL) according to the manufacturer's instructions. The sensitivity of the assay was 12 fmol per assay well. The cellular cAMP level was presented as pmol per 10^6 cells.

Measurement of cyclic nucleotide phosphodiesterase (PDE) activity T cells were cultured under the indicated conditions, and were lyzed in the buffer containing 20 mM Tris–HCl (pH 7.4), 1 mM ethylenediamine tetraacetic acid, 1 μg aprotinin per ml, 1 μg pepstatin per ml, 1 μg leupeptin per ml, 15 mM benzamidine, and 3.75 mM β-mercaptoethanol. PDE activity of the cell lysate was assayed as described (Robicsek *et al*, 1991) using 1 mM [2,8-^{3}H] cAMP (30 Ci per mmol)

(Amersham) as a substrate. The assays were performed in 40 mM Tris–HCl (final pH 8.0), 10 mM $MgCl_2$ at 37°C for 10 min, and PDE activity was presented as pmol cAMP hydrolyzed per min per mg protein.

Measurement of adenylate cyclase (AC) activity The T cell lysate was centrifuged at 23,600 × *g* for 10 min. The pellet was used as a particulate fraction for AC assays as described (Salomon *et al*, 1974; Choi *et al*, 1992). The AC activity of the particulate fraction was measured at 37°C for 10 min in 20 mM Tris–HCl (pH 7.4), 1 mM [α-^{32}P]adenosine triphosphate (30 Ci per mmol) (Amersham), 1 mM [^{3}H]cAMP, 1 mM 3-isobutyl-1-methylxanthine, 5 mM $MgCl_2$, 0.2 mM ethyleneglycol-bis-(β-aminoethylether)-*N,N,N′,N′*-tetraacetic acid, 20 mM creatine phosphate, and 100 units creatine phosphokinase per ml. AC activity was presented as pmol cAMP formed per min per mg protein.

Plasmids and transfection pCAT3-basic vector carrying two SV40 poly(A) signals, one downstream of the CAT reporter gene, and the other upstream of the multicloning site was purchased from Promega (Madison, WI). The plasmid IL-2-CAT, which contains human IL-2 promoter (bp −541 to +42 relative to the transcriptional start site), was generated by PCR using human genomic DNA (Clontech, Heidelberg, Germany) and primers based on the reported sequence (Siebenlist *et al*, 1986), and cloned into *Nhe*I/*Bgl*II site of pCAT3-basic vector. The plasmids IFN-γ-CAT, IL-4-CAT, or IL-5-CAT containing the promoters of IFN-γ (bp −337 to +64), IL-4 (bp −418 to +50), or IL-5 (bp −511 to +4), respectively, were generated as described (Penix *et al*, 1993; Paliogianni *et al*, 1996; Mori *et al*, 1997). The entire cloned regions were sequenced by the chain termination method and found to be identical to the reported genomic sequences (Siebenlist *et al*, 1986; Taya *et al*, 1982; Tanabe *et al*, 1987; Arai *et al*, 1989). Transfection of Jurkat cells was carried out by the DEAE-dextran method as described (Lee *et al*, 1993). Cells (10^7) were incubated with 10 μg DNA per ml and 350 μg DEAE-dextran per ml (Pharmacia) in Tris-buffered saline for 30 min at room temperature. To decrease variations in transfection efficiency, cells were transfected in single batches, which were then separated into different drug treatment groups. Cells were washed with Tris-buffered saline, and incubated with DMEM/F12. After 24 h, cells were washed, preincubated with anti-mycotics for 30 min, and seeded to anti-CD3/CD28-coated plates at a density of 4×10^5 cells per well, and cultured in the presence or absence of the respective anti-mycotics in 200 μl of DMEM/F12. After 16 h, the cells were harvested and lyzed by three freeze/thaw cycles. The cell lysate was centrifuged and supernatant was assayed for CAT expression by CAT-ELISA (Roche Diagnostics, Tokyo, Japan) according to the manufacturer's instructions. Total protein amount was measured by a Bradford microassay procedure (Bio-Rad, Hercules, CA). The expression of various CAT plasmids was presented as pg CAT enzyme synthesized per μg total protein. pCAT3-control vector (Promega) containing SV40 early promoter and enhancer sequences was used as a positive control, and promoterless pCAT3-basic vector was used as a negative control.

Assays of cAMP-dependent protein kinase [protein kinase A (PKA)] The T cell lysate was assayed for the activity of PKA using an ELISA kit (Medical and Biological Laboratories, Nagoya, Japan) by examining the phosphorylation of plate-bound peptide substrate in the presence or absence of 2 μM cAMP for 10 min at room temperature. The plates were sequentially incubated with biotinylated antibody to the phosphorylated substrate, peroxidase-conjugated streptavidin, *o*-phenylenediamine, then the reaction was stopped, and optical density at 492 nm was read. The PKA activity was expressed as an activity ratio, which is defined as the optical density in the absence of exogenous cAMP divided by the optical density in the presence of cAMP added to the assay.

Statistical analyses Student's t-test was used for the comparison of mean amounts in AD and normal groups in **Table I**. One-way analysis of variance with Dunnet's multiple comparison test was used for the data in **Figs 1 and 2**. One-way analysis of variance with Scheffe's multiple comparison test was used for the data in **Figs 3**(***a–d***) and (***b***, ***c***) and **Table II** and **III**. Spearman's correlation coefficient was determined for the data in **Fig 5**. A value of $p < 0.05$ was considered significant.

RESULTS

The effects of anti-mycotics on anti-CD3/CD28-induced IL-4, IL-5, IFN-γ, and IL-2 secretion First, the effects of various anti-mycotics were examined on anti-CD3/CD28-induced secretion of Th1 and Th2 cytokines by T cells from AD patients and normal donors. The anti-CD3/CD28-induced IL-4 secretion was significantly higher in AD patients than that in normal donors (**Table I**). Azole derivatives, ketoconazole, itraconazole, and miconazole and nonazole terbinafine hydrochloride and tolnaftate reduced the anti-CD3/CD28-induced IL-4 secretion in AD patients' T cells in a concentration-dependent manner (**Fig 1*a***); percentage inhibition by 1 μM of ketoconazole, itraconazole, miconazole, terbinafine hydrochloride, or tolnaftate was 60%, 58%, 58%, 40%, and 38%, respectively. Thus the inhibitory effects of azole derivatives ketoconazole, itraconazole, and miconazole were mostly equivalent, and were greater than those of nonazole terbinafine hydrochloride and tolnaftate. These anti-mycotics also reduced the anti-CD3/CD28-induced IL-4 secretion in normal donors' T cells to the extents comparable with those in AD patients' T cells (**Fig 1*a***). The percentage inhibition by 1 μM of ketoconazole, itraconazole, miconazole, terbinafine hydrochloride, or tolnaftate was 62%, 58%, 57%, 42%, or 40%, respectively, in normal donors' T cells. The anti-CD3/CD28-induced IL-5 secretion by T cells was also significantly higher in AD patients than in normal donors (**Table I**). The azole and nonazole anti-mycotics also reduced the anti-CD3/CD28-induced IL-5 secretion, and the magnitude of the reduction was equivalent in AD patients and in normal donors (**Fig 1*b***); percentage inhibition by 1 μM of ketoconazole, itraconazole, miconazole, terbinafine hydrochloride, or tolnaftate was 70%, 69%, 69%, 50%, or 49%, respectively, in AD patients compared with 72%, 69%, 68%, 49%, or 48%, respectively, in normal donors. Thus the anti-mycotics significantly reduced anti-CD3/CD28-induced IL-4 and IL-5 secretion of both AD and normal T cells, and azole derivatives were more inhibitory than nonazole anti-mycotics. None of ketoconazole, itraconazole, miconazole, terbinafine hydrochloride, and tolnaftate reduced the viability of T cells, which was ⩾ 95% after 48 h of incubation with the anti-mycotics, as examined by the trypan blue dye exclusion test.

The anti-CD3/CD28-induced IFN-γ secretion in AD patients was not significantly different from that in normal donors (**Table I**), and the secretion was not significantly altered by azole and nonazole anti-mycotics (**Fig 1*c***). Though 1 μM of azole derivatives, ketoconazole, itraconazole, and miconazole appeared to increase the IFN-γ secretion slightly (9–12%) in AD patients and

Table I. Anti-CD3/CD28-induced Th2/Th1 cytokine secretion and anti-CD3/CD28-induced increase of cAMP level and of PKA activity in T cells from AD patients and normal donors[a]

	AD (n = 15)	Normal (n = 14)
IL-4 (pg per ml)	235.6 ± 24.2[b]	112.5 ± 12.3
IL-5 (pg per ml)	461.3 ± 42.1[b]	221.5 ± 23.6
IFN-γ (pg per ml)	812.5 ± 92.3	1005.2 ± 122.4
IL-2 (pg per ml)	1205.3 ± 141.4	1376.5 ± 151.2
cAMP (pmol per 10^6 cells)	5.4 ± 0.6[b]	2.1 ± 0.3
PKA (–cAMP/+cAMP)[c]	0.43 ± 0.04[b]	0.17 ± 0.02

[a]T cells from AD patients or normal donors were incubated in anti-CD3 plus anti-CD28-precoated plates. The intracellular cAMP level and cell lysate PKA activity were analyzed after 5 min, whereas cytokine secretion was assayed after 48 h, and the background values without anti-CD3/CD28 were subtracted. Background IL-4 secretion without anti-CD3/CD28 was less than detectable level in both AD and normal T cells. The background IL-5, IFN-γ, or IL-2 secretion was 5.4 ± 1.6 pg per ml, 6.5 ± 1.1 pg per ml, or 11.3 ± 2.6 pg per ml in AD T cells (mean ± SEM, n = 15), whereas 4.5 ± 0.7 pg per ml, 7.8 ± 1.4 pg per ml, or 11.9 ± 2.1 pg per ml in normal T cells (mean ± SEM, n = 14), respectively. The background cAMP level was 1.8 ± 0.2 or 2.0 ± 0.3 pmol cAMP per 10^6 cells in AD or normal T cells, respectively.

[b]$p < 0.05$ *vs* values of normal donors, by Student's t test.

[c]The PKA activity was expressed as an activity ratio, which is defined as the optical density in the absence of exogenous cAMP divided by the optical density in the presence of cAMP added to the assay. The background PKA activity was 0.20 ± 0.04 or 0.21 ± 0.04 in AD or normal T cells, respectively.

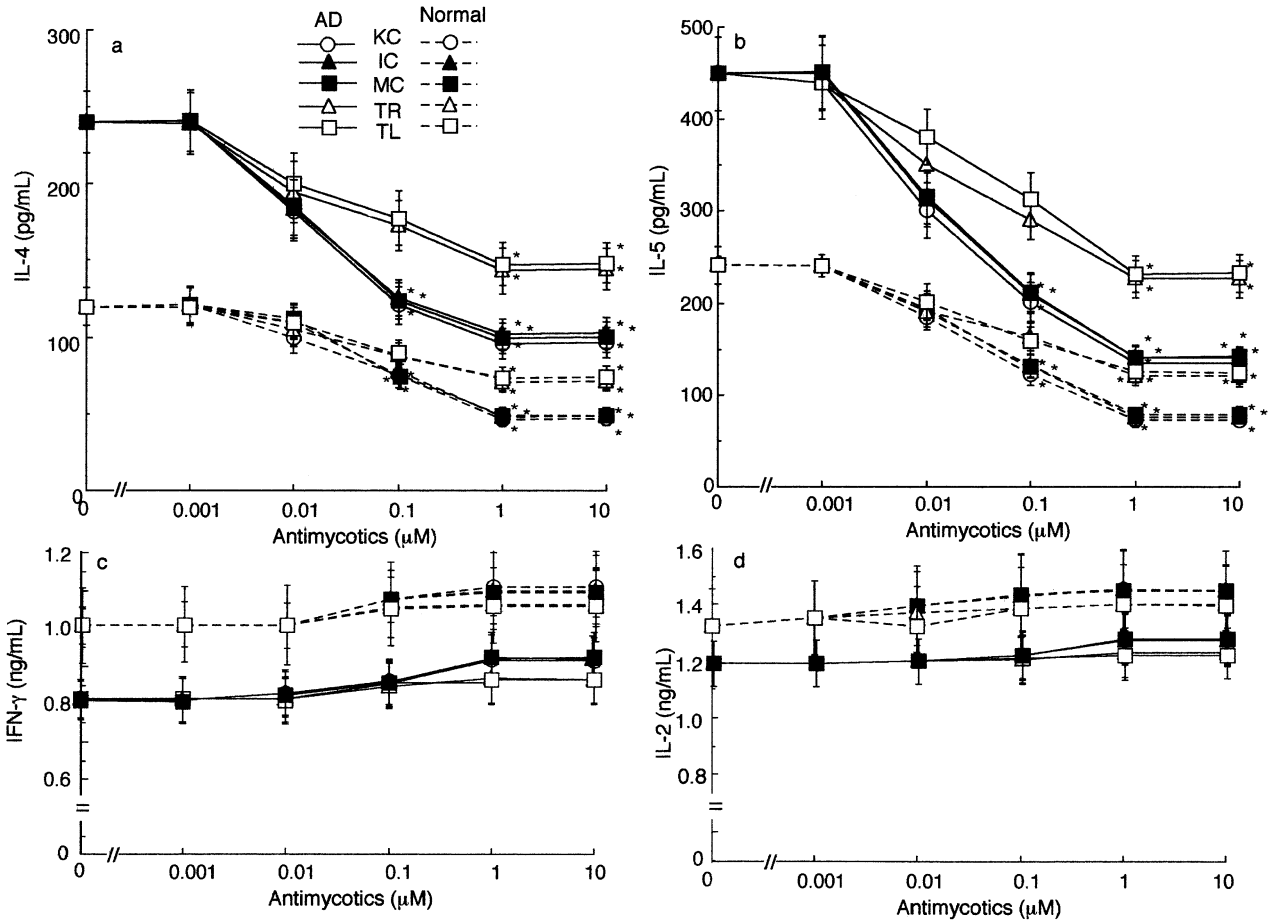

Figure 1. Concentration-dependency for the effects of various anti-mycotics on anti-CD3/CD28-induced secretion of Th2 and Th1 cytokines. T cells from an AD patient or normal donor were preincubated for 30 min with medium alone or with medium containing anti-mycotics at indicated doses, and seeded to anti-CD3 plus anti-CD28-precoated plates, and cultured in the presence or absence of respective anti-mycotics for another 48 h. The culture supernatants were assayed for IL-4 (*a*), IL-5 (*b*), IFN-γ (*c*), and IL-2 (*d*) by ELISA, and background cytokine secretion without anti-CD3/CD28 was subtracted. Values are the mean ± SD of triplicate cultures. The background IL-4 secretion without anti-CD3/CD28 was less than detectable level in both AD and normal T cells. The background IL-5, IFN-γ, or IL-2 secretion was 5.7 ± 0.6 pg per ml, 6.4 ± 0.7 pg per ml, or 10.3 ± 1.0 pg per ml (mean ± SD of triplicate) in AD T cells whereas 4.3 ± 0.4 pg per ml, 7.9 ± 0.7 pg per ml, or 11.2 ± 1.1 pg per ml in normal T cells, respectively. *$p < 0.05$ *vs* control cultures with anti-CD3/CD28 alone and without anti-mycotics, by one-way analysis of variance with Dunnet's multiple comparison test. The data represent five separate experiments using T cells from five different AD patients and five different normal donors.

normal donors, the increase was not significant. The anti-CD3/CD28-induced IL-2 secretion by T cells in AD patients was not significantly different from that in normal donors (**Table I**), and the secretion was not significantly altered by the anti-mycotics. Thus azole and nonazole anti-mycotics did not affect anti-CD3/CD28-induced secretion of Th1 cytokines. We next examined if the anti-mycotics may alter mRNA expression for Th1 and Th2 cytokines.

The effects of anti-mycotics on anti-CD3/CD28-induced mRNA expression for Th1 and Th2 cytokines As analyzed by reverse transcription–PCR, ketoconazole, terbinafine hydrochloride, and tolnaftate reduced anti-CD3/CD28-induced IL-4 and IL-5 mRNA expression at 6 h incubation of T cells from AD patients (**Fig 6**). The magnitude of the inhibition by an azole derivative ketoconazole on IL-4 and IL-5 mRNA expression was higher than that by nonazole terbinafine hydrochloride and tolnaftate; percentage inhibition of IL-4/β-actin ratio by ketoconazole, terbinafine hydrochloride, or tolnaftate was 67%, 44%, or 39%, and that of IL-5/β-actin was 62%, 40%, or 41%, respectively (**Fig 6*b***). In contrast, none of the anti-mycotics altered anti-CD3/CD28-induced IFN-γ and IL-2 mRNA expression (**Fig 6*a***, ***b***). The other azole derivatives, itraconazole and miconazole reduced IL-4 and IL-5 mRNA expression to an extent equivalent to that of ketoconazole without altering that of IFN-γ and IL-2 (data not shown). In normal donors' T cells, the azole and nonazole anti-mycotics reduced anti-CD3/CD28-induced IL-4 and IL-5 mRNA expression and did not alter that of IFN-γ and IL-2 (data not shown). These results in mRNA levels paralleled those in protein secretion for Th1 and Th2 cytokines (**Fig 1**), suggesting the pretranslational downregulation of Th2 cytokine production by the anti-mycotics. We then examined if these anti-mycotics may exert their action at the transcriptional level by analyzing the effects of the anti-mycotics on the promoter activities for Th1 and Th2 cytokines.

The effects of anti-mycotics on promoter activities for Th1/Th2 cytokines Human Jurkat T cells were transiently transfected with plasmids containing human IL-2, IFN-γ, IL-4, or IL-5 promoters driving CAT reporter gene. The transfected cells were preincubated with ketoconazole, terbinafine hydrochloride, or tolnaftate for 30 min, washed, and incubated with anti-CD3/CD28 in the presence of respective anti-mycotics. The promoter activity was assessed by the expression of CAT enzyme. The attempt to transfect fresh peripheral blood T cells was unsuccessful (data not shown). As shown in **Table II**, the anti-mycotics reduced

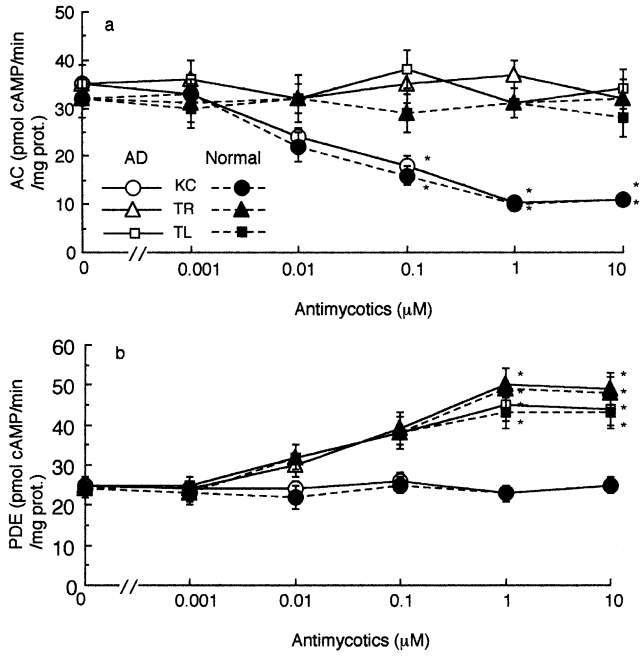

Figure 2. Concentration-dependency for the effects of various anti-mycotics on AC and PDE activities. (*a*) AC activities; (*b*) PDE activities. The particulate fraction of freshly isolated peripheral blood T cells from an AD patient and normal donor was assayed for AC activity (*a*), and the whole cell lysate was assayed for PDE activity (*b*) in the presence or absence of indicated doses of ketoconazole, terbinafine hydrochloride, or tolnaftate. Values are the mean ± SD of triplicate assays. The data represent five separate experiments using T cells from five different AD patients and five different normal donors. *$p < 0.05$ *vs* values without anti-mycotics, by one-way analysis of variance with Dunnet's multiple comparison test.

the anti-CD3/CD28-induced IL-4 and IL-5 promoter activities, and ketoconazole was more inhibitory than terbinafine hydrochloride and tolnaftate. In contrast, these anti-mycotics did not alter the anti-CD3/CD28-induced IFN-γ and IL-2 promoter activities. The other azole derivatives itraconazole and miconazole also reduced anti-CD3/CD28-induced IL-4 and IL-5 promoter activities by the magnitude comparable with that of ketoconazole, without altering IFN-γ and IL-2 promoter activities (data not shown). These results in transfection assays are consistent with those in mRNA expression (**Fig 6**) and protein secretion (**Fig 1**) for Th1/Th2 cytokines. It is thus suggested that the anti-mycotics may reduce Th2 cytokine production at the transcriptional level, although post-transcriptional regulation is also implicated.

CAMP-induced reversal from the anti-mycotic-induced inhibition on IL-4 and IL-5 production It is known that anti-mycotics, especially azole derivatives modulate a variety of intracellular signaling pathways via regulating intracellular enzyme activity (Beetens *et al*, 1986; Janssen *et al*, 1989; Stalla *et al*, 1989; Hegemann *et al*, 1993). Thus the anti-mycotics may repress anti-CD3/CD28-induced IL-4 and IL-5 production by inhibiting certain signaling pathways required for the production. To identify the target signal(s) for the anti-mycotics, we examined if several signal-inducers may counteract the anti-mycotic-induced repression of IL-4 and IL-5 production. The signal inducers were cAMP analog Bt_2cAMP which activates PKA, cGMP analog Bt_2cGMP activating cGMP-dependent protein kinase, and diacylglycerol analog OAG activating protein kinase C. As shown in **Fig 3**, these agents were added at low concentrations that did not alter anti-CD3/CD28-induced IL-4 and IL-5 production in the absence of anti-mycotics. Bt_2cAMP reversed the ketoconazole-mediated repression of IL-4 and IL-5 secretion in AD patients' T cells (**Fig 3*a*, *b***) and of IL-4 and IL-5 promoter activities in Jurkat T cells (**Fig 3*c*, *d***). As the intracellular cAMP is synthesized by AC and hydrolyzed by PDE, cAMP can be increased by the stimulation of AC and/or inhibition of PDE. The ketoconazole-mediated inhibition of IL-4 and IL-5 secretion and of promoter activities were also reversed by cAMP-elevating agents, AC activator forskolin (1 μM) or PDE inhibitor 3-isobutyl-1-methylxanthine (50 μM) (data not shown). Bt_2cAMP (**Fig 3*e***), forskolin, or 3-isobutyl-1-methylxanthine (data not shown) also counteracted the ketoconazole-mediated inhibition of IL-4 and IL-5 mRNA expression in AD patients' T cells. These results indicate that the cAMP/PKA signaling pathway may be involved in the ketoconazole-mediated inhibition of IL-4 and IL-5 production. On the other hand, Bt_2cGMP or OAG did not counteract the ketoconazole-induced inhibition on IL-4 and IL-5 secretion (**Fig 3*a*, *b***), promoter activities (**Fig 3*c*, *d***), and mRNA expression (**Fig 3*e***), indicating that protein kinase C or cGMP-dependent protein kinase may not be involved in the ketoconazole-mediated inhibition of IL-4 and IL-5 production. The specific PKA inhibitor H-89 reduced anti-CD3/CD28-induced IL-4 secretion (**Fig 3*a***) or promoter activity (**Fig 3*c***) by 79% or 80%, and IL-5 secretion (**Fig 3*b***) or promoter activity (**Fig 3*d***) by 81% or 80% compared with controls, respectively, suggesting that cAMP/PKA signaling pathway may be required for anti-CD3/CD28-induced IL-4 and IL-5 production. In the presence of H-89, ketoconazole did not further reduce the anti-CD3/CD28-induced IL-4 and IL-5 secretion and promoter activities, which confirms that cAMP/PKA pathway may be the main target for the ketoconazole-mediated inhibition of IL-4 and IL-5 production. On the other hand, H-89 did not alter anti-CD3/CD28-induced IFN-γ and IL-2 secretion, mRNA expression, and promoter activities (data not shown), indicating that PKA may not be required for the production of Th1 cytokines. Bt_2cAMP or cAMP-elevating agents also counteracted the effects of itraconazole, miconazole, terbinafine hydrochloride, and tolnaftate on IL-4 and IL-5 secretion and mRNA expression in AD patients' T cells and on IL-4 and IL-5 promoter activities in Jurkat T cells (data not shown). In normal donors' T cells, Bt_2cAMP or cAMP-elevating agents also counteracted the effects of ketoconazole, itraconazole, miconazole, terbinafine hydrochloride, and tolnaftate on IL-4 and IL-5 secretion and mRNA expression induced by anti-CD3/CD28, and the anti-mycotics did not further reduce the IL-4 and IL-5 production of normal donors' T cells in the presence of H-89 (data not shown). These results suggest that the anti-mycotic-induced inhibition of IL-4 and IL-5 production may be attributable to the inhibition of cAMP/PKA signaling pathway in both AD patients' and normal donors' T cells. We then examined whether the anti-mycotics may alter cAMP level in anti-CD3/CD28-stimulated T cells.

The effects of anti-mycotics on cAMP level in anti-CD3/CD28-stimulated T cells We examined the kinetics of cAMP level of T cells after incubation with anti-CD3/CD28 (**Fig 4*a***). Though the basal cAMP level was not different between AD patients' and normal donors' T cells, the cAMP level of AD T cells increased 4-fold above the basal level 5 min after the anti-CD3/CD28 stimulus, whereas that of normal T cells increased 2-fold. The anti-CD3/CD28-induced increase of cAMP at 5 min in AD T cells was significantly higher than that in normal T cells (**Table I**). In both AD and normal T cells, the anti-CD3/CD28-induced increase of cAMP correlated with anti-CD3/CD28-induced IL-4 and IL-5 secretion, although the correlation was greater in AD T cells than in normal T cells (**Fig 5**). On the other hand, there was no significant correlation between anti-CD3/CD28-induced cAMP increase and IFN-γ or IL-2 secretion by anti-CD3/CD28. These results indicate that the anti-CD3/CD28-induced increase of cAMP may be required for IL-4 and IL-5 production, whereas dispensable for that of IFN-γ and IL-2. Ketoconazole, itraconazole, or miconazole reduced the anti-CD3/CD28-induced increase of cAMP by 75, 74, or 73%, whereas terbinafine hydrochloride or tolnaftate reduced the increase by 46 or 44% in AD T cells,

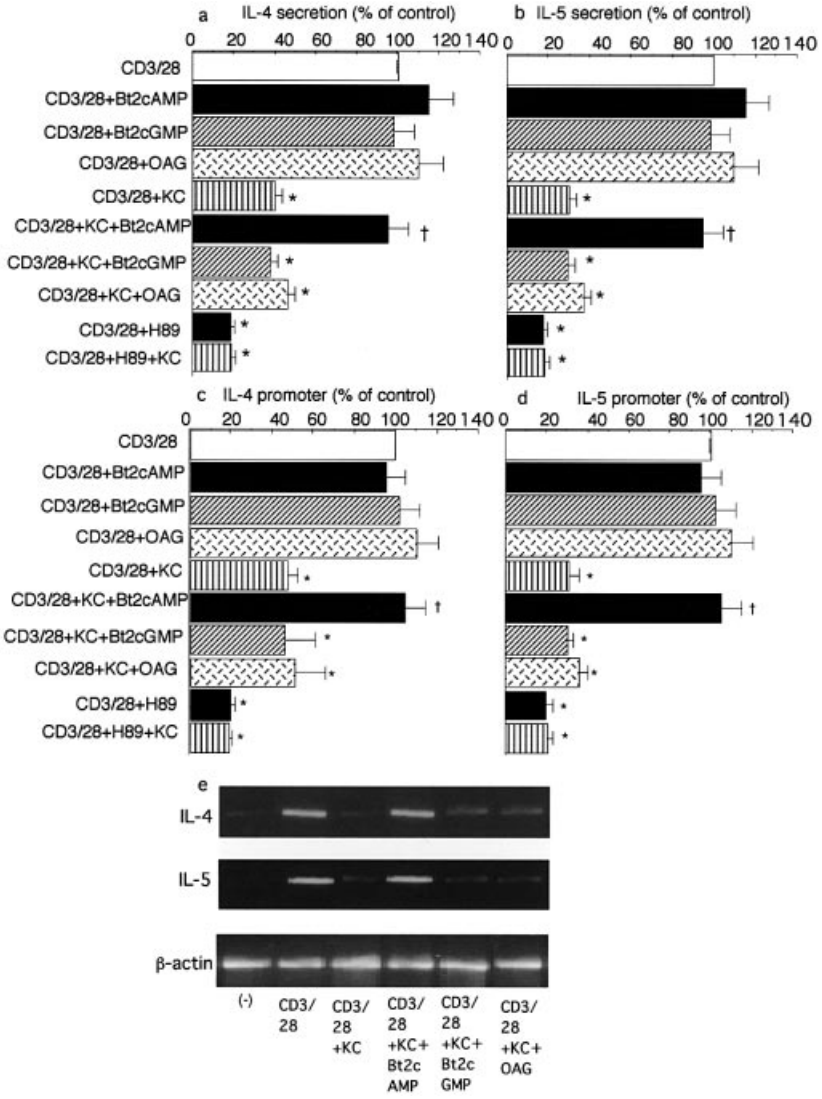

Figure 3. Bt_2cAMP-mediated reversal from ketoconazole-induced inhibition on IL-4 and IL-5 secretion, promoter activities, and mRNA expression induced by anti-CD3/CD28. (*a*, *b*) T cells from five different AD patients were preincubated for 30 min with or without Bt_2cAMP 50 μM, Bt_2cGMP 50 μM, OAG 100 ng per ml, or H-89 0.1 μM in the presence or absence of ketoconazole 1 μM, then seeded on to anti-CD3 plus anti-CD28-precoated plates, and cultured for another 48 h in the presence or absence of the above-mentioned agents. Cytokine secretion was analyzed by ELISA, and background secretion without anti-CD3/CD28 was subtracted. (*c*, *d*) Jurkat T cells were transfected with IL-4 promoter-CAT reporter (*c*) or IL-5 promoter-CAT reporter plasmid (*d*). After 24 h, the transfected cells were preincubated with indicated agents as above, then incubated with plate-bound anti-CD3/CD28 in the presence or absence of respective agents. After 16 h, IL-4 or IL-5 promoter activity was assayed by CAT expression of the cell lysate. The data are shown as percent *vs* the values in control cultures with anti-CD3/CD28 alone, and represent the mean ± SEM of five separate experiments. The background IL-4 secretion and IL-4 and IL-5 promoter activities without anti-CD3/CD28 were less than detectable level. The background IL-5 secretion was 5.5 ± 0.6 pg per ml (mean ± SEM, n = 5). The anti-CD3/CD28-induced secretion of IL-4 or IL-5 without the other agents was 232.5 ± 26.3 or 447.3 ± 54.6 pg per ml (mean ± SEM, n = 5), respectively. The anti-CD3/28-induced IL-4 or IL-5 promoter activity was 17.5 ± 3.5 or 19.5 ± 2.3 pg CAT per μg protein (mean ± SEM, n = 5), respectively. *$p < 0.05$ *vs* cultures with anti-CD3/CD28 alone and †$p < 0.05$ *vs* cultures with anti-CD3/CD28 plus ketoconazole, by one-way analysis of variance with Scheffe's multiple comparison test. (*e*) T cells from an AD patient were preincubated with indicated agents, and incubated with anti-CD3 plus anti-CD28 as described above. After 6 h, RNA was extracted and reverse transcription–PCR products were analyzed by electrophoresis. The data represent five separate experiments using T cells from five different AD patients.

respectively (**Fig 4*b***). Thus the inhibitory effects on cAMP by azole derivatives ketoconazole, itraconazole, and miconazole were mostly equivalent and were greater than those by nonazole terbinafine hydrochloride and tolnaftate. The anti-CD3/CD28 enhanced PKA activity at 5 min in AD and normal T cells, and the enhancement was greater in AD T cells than in normal T cells (**Table I**), which paralleled the increase of cAMP. The anti-mycotics suppressed the anti-CD3/CD28-induced activation of PKA in AD patients' T cells (**Fig 4*c***) in parallel with the reduction of cAMP (**Fig 4*b***). The magnitude of the suppression of PKA was greater in azole ketoconazole, itraconazole, and miconazole than in nonazole terbinafine hydrochloride and tolnaftate (**Fig 4*c***), which paralleled the magnitude of the reduction of cAMP by each agent (**Fig 4*b***). In normal donors' T cells, the anti-mycotics suppressed the anti-CD3/CD28-induced increase of cAMP and of PKA activity, and azole derivatives were more suppressive than nonazoles (data not shown). Thus the anti-mycotics suppressed the cAMP signal induced by anti-CD3/CD28, and the suppressive effects of azole derivatives ketoconazole, itraconazole, and miconazole were greater than nonazole terbinafine hydrochloride or tolnaftate. As the reduction of cAMP level can be mediated by the inhibition of AC and/or stimulation of PDE, we examined if the anti-mycotics may alter AC and/or PDE activity.

The effects of anti-mycotics on AC and PDE activities of T cells As shown in **Table III**, anti-CD3/CD28 increased AC activity of AD or normal T cells 4.5-fold or 2.5-fold above the basal level, respectively. Though the basal AC activity was not different between AD and normal T cells ($p > 0.10$ by Student's t test), the anti-CD3/CD28-promoted AC activity in AD T cells was significantly higher than that in normal T cells ($p < 0.05$ by Student's t test). The anti-CD3/CD28-induced increase of AC was suppressed by ketoconazole in both AD and normal T cells. Ketoconazole also reduced the basal AC activity in the absence of anti-CD3/CD28 in both AD and normal T cells. Itraconazole and miconazole also suppressed the basal and anti-CD3/CD28-promoted AC activity by the magnitude comparable with that of ketoconazole in both AD and normal T cells (data not shown). In contrast, terbinafine hydrochloride and tolnaftate did not alter the basal and anti-CD3/CD28-promoted AC activity in either AD or normal T cells. The anti-CD3/CD28 also enhanced PDE activity of AD or normal T cells 2.0-fold or 1.5-fold above the basal level 5 min after the stimuli, respectively, and terbinafine hydrochloride and tolnaftate promoted the basal and anti-CD3/CD28-enhanced PDE activities in both AD and normal T cells, whereas those were not altered by ketoconazole (**Table III**), itraconazole, and miconazole (data not shown). These results suggest that

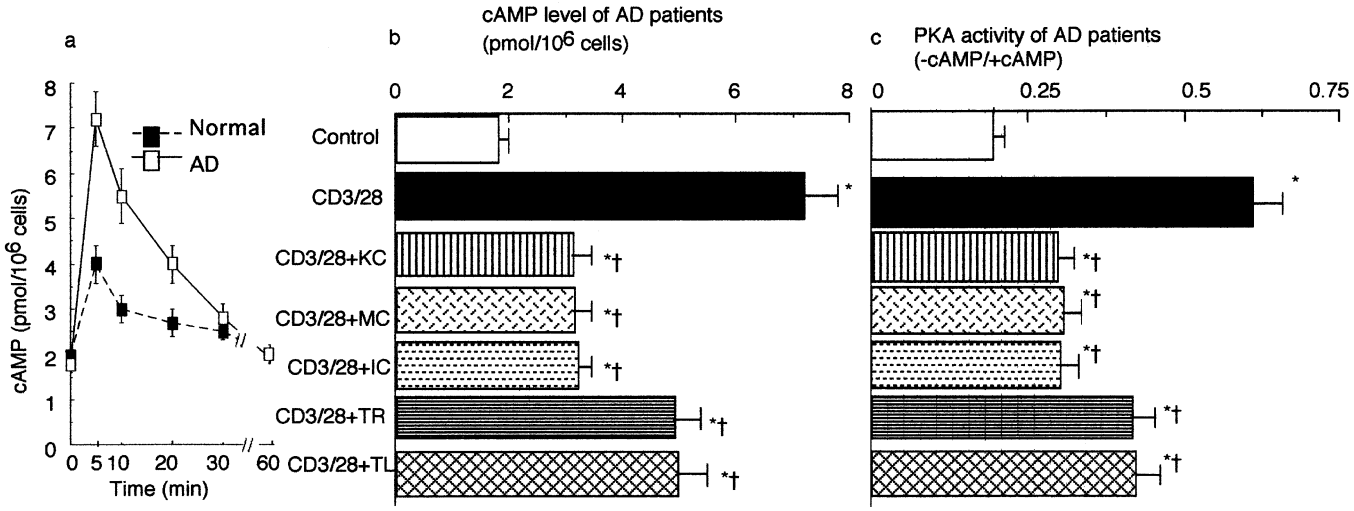

Figure 4. Kinetics of intracellular cAMP level after anti-CD3/CD28 stimulus and anti-mycotic-mediated effects on anti-CD3/CD28-induced increase of cAMP and of PKA activity in T cells. (*a*) T cells from an AD patient and a normal donor were incubated with plate-bound anti-CD3 plus anti-CD28. Intracellular cAMP level was analyzed at the indicated time points. The mean ± SD of triplicate cultures is shown. The data are representative of five separate experiments using T cells from five different AD patients and five different normal donors. (*b*) T cells from 15 AD patients were preincubated with or without ketoconazole, itraconazole, miconazole, terbinafine hydrochloride, or tolnaftate (each 1 μM) for 30 min, and seeded on to anti-CD3 plus CD28-precoated or noncoated plates, and incubated in the presence or absence of respective anti-mycotics. After 5 min, the cells were harvested and assayed for cAMP level (*b*) or PKA activity (*c*). The data are mean ± SEM (n = 15). *$p < 0.05$ *vs* values without anti-CD3/CD28, and †$p < 0.05$ *vs* values with anti-CD3/CD28 alone, by one-way analysis of variance with Scheffe's multiple comparison test.

Table II. The effects of anti-mycotics on the promoter activities for Th2 and Th1 cytokines[a]

	CAT expression (pg CAT per μg protein)			
	IL-4	IL-5	IFN-γ	IL-2
CD3/CD28	19.6 ± 2.2	17.4 ± 1.8	55.8 ± 5.3	65.6 ± 6.2
CD3/CD28+ ketoconazole	7.0 ± 0.8[b]	5.9 ± 0.6[b]	56.1 ± 6.1	66.3 ± 6.1
CD3/CD28+ terbinafine hydrochloride	10.5 ± 2.1[bc]	9.8 ± 1.1[bc]	56.0 ± 5.8	65.8 ± 6.9
CD3/CD28+ tolnaftate	11.3 ± 1.6[bc]	9.9 ± 1.0[bc]	55.6 ± 5.9	66.1 ± 6.8

[a]Jurkat T cells were transiently transfected with IL-4, IL-5, IFN-γ, or IL-2 promoter-CAT reporter plasmids, and preincubated with medium alone or with ketoconazole, terbinafine hydrochloride, or tolnaftate (each 1 μM) for 30 min, and seeded to anti-CD3 plus anti-CD28-precoated plates, and cultured in the presence or absence of respective anti-mycotics for 16 h. The activity of each promoter was assessed by CAT expression of the cell lysate. The data are mean ± SD of triplicate assays, and are representative of five separate experiments. The CAT expression by each cytokine promoter-CAT reporter plasmid without anti-CD3/CD28 was less than detectable level. The CAT expression by pCAT3-control vector without stimuli was 192.5 ± 17.8 pg CAT/μg protein, and that by pCAT3-basic vector was less than detectable level.

[b]$p < 0.05$ *vs* values with anti-CD3/CD28 alone, and

[c]$p < 0.05$ *vs* values with anti-CD3/CD28 plus ketoconazole, by one-way analysis of variance with Scheffe's multiple comparison test.

ketoconazole, itraconazole, and miconazole may suppress AC activity, whereas terbinafine hydrochloride and tolnaftate may enhance PDE activity and thus may reduce cAMP levels in anti-CD3/CD28-stimulated T cells from AD patients and normal donors.

To know the direct effects of the anti-mycotics on AC or PDE activity, we directly added each anti-mycotic to the reaction mixture of AC or PDE assay. Ketoconazole reduced whereas terbinafine hydrochloride and tolnaftate did not alter AC activity of T cell particulate fractions; the percent inhibition *vs* controls by 1 μM ketoconazole was 72% and 70% in AD and normal T cells, respectively (**Fig 2*a***). In contrast, terbinafine hydrochloride and tolnaftate enhanced, whereas ketoconazole did not alter PDE activity of T cell lysates (**Fig 2*b***); the percent stimulation *vs* controls by 1 μM terbinafine hydrochloride or tolnaftate was 95% or 80% in AD T cells and 92% or 72% in normal T cells, respectively. The other azole derivatives itraconazole and miconazole reduced AC activities of particulate fractions from AD and normal T cells by the magnitude similar to that of ketoconazole without altering PDE activity (data not shown). These results suggest that azole derivatives ketoconazole, itraconazole, and miconazole may suppress AC activity, whereas nonazole terbinafine hydrochloride and tolnaftate may stimulate PDE activity and thus suppress anti-CD3/CD28-induced increase of cAMP in AD and normal T cells. The suppression of cAMP signal may inhibit the activation of PKA, which may result in the repression of IL-4 and IL-5 production. AC-inhibiting ketoconazole, itraconazole, and miconazole were more effective for the reduction of cAMP, and of IL-4 and IL-5 production than PDE-stimulating terbinafine hydrochloride and tolnaftate. On the other hand, the anti-mycotic-induced reduction of cAMP may not reduce IFN-γ and IL-2 production in both AD and normal T cells (**Fig 1**), indicating that the cAMP signal by anti-CD3/CD28 may not be required for the IFN-γ and IL-2 production.

DISCUSSION

In this study, anti-mycotics reduced IL-4 and IL-5 production in anti-CD3/CD28-stimulated AD and normal T cells without altering that of IFN-γ and IL-2. A previous study reported the discrepant results; itraconazole and miconazole nondifferentially inhibited phytohemagglutinin-induced mRNA accumulation for IL-2, IL-4, and IFN-γ in human peripheral blood mononuclear cells (Friccius *et al*, 1992). Those nondifferential effects by itraconazole and miconazole, however, occurred only at high doses (10 μg per ml, i.e., 14.2 μM for itraconazole and 24 μM for miconazole) and after extended incubation (24 h), whereas 6 h incubation with these agents at 1 μM differentially suppressed anti-CD3/CD28-induced mRNA expression for IL-4 and IL-5, and not for IL-2 or IFN-γ in our present study (**Fig 6**). Thus the mechanism for the nondifferential inhibition by itraconazole and

Table III. The effects of anti-mycotics on AC and PDE activities of T cells[a]

	AC activity[b]		PDE activity[b]	
	CD3/CD28 (–)	CD3/CD28 (+)	CD3/CD28 (–)	CD3/CD28 (+)
AD patients				
None	33.8 ± 5.0	153.1 ± 15.9	18.9 ± 2.1	37.0 ± 3.6
Ketoconazole	11.3 ± 1.5 (–67)[c]	55.1 ± 6.7 (–64)[c]	19.0 ± 1.9 (0)	35.6 ± 3.8 (–4)
Terbinafine hydrochloride	34.1 ± 4.6 (1)	150.2 ± 16.1 (–2)	34.5 ± 4.6 (83)[c]	69.1 ± 8.9 (87)[c]
Tolnaftate	34.0 ± 5.1 (1)	148.4 ± 16.1 (–3)	33.2 ± 3.5 (76)[c]	65.3 ± 8.6 (76)[c]
Normal donors				
None	39.5 ± 4.5	81.3 ± 8.4	18.1 ± 1.9	27.1 ± 3.2
Ketoconazole	16.8 ± 2.8 (–57)[c]	36.3 ± 4.7 (–55)[c]	17.0 ± 1.9 (–6)	25.6 ± 3.5 (–6)
Terbinafine hydrochloride	39.1 ± 4.0 (0)	84.2 ± 9.1 (4)	31.9 ± 4.9 (72)[c]	50.1 ± 6.1 (85)[c]
Tolnaftate	38.9 ± 3.6 (–1)	82.4 ± 9.0 (1)	30.8 ± 3.7 (70)[c]	47.2 ± 5.0 (74)[c]

[a]T cells from 15 AD patients and 14 normal donors were preincubated with medium alone or with ketoconazole, terbinafine hydrochloride, or tolnaftate (each 1 μM) for 30 min, and seeded to anti-CD3 plus anti-CD28-precoated (+) or noncoated (–) plates, and incubated in the presence or absence of respective anti-mycotics for 5 min. T cells were then harvested and assayed for AC and PDE activities. The data are mean ± SEM (n = 15 for AD patients; n = 14 for normal donors). The values in parentheses are the percent decrease or increase *vs* controls without anti-mycotics.

[b]pmol cAMP per min per mg protein.

[c]$p < 0.05$ *vs* controls without anti-mycotics, by one–way analysis of variance with Scheffe's multiple comparison test.

miconazole in Friccius *et al* (1992) may differ from that for the Th2-preferential inhibition in our study. The Th2-preferential inhibition in our study was mediated by reducing anti-CD3/CD28-induced cAMP signal. The inhibition of cAMP by azole derivatives ketoconazole, itraconazole, and miconazole were mediated by the inhibition of AC, whereas those of nonazole terbinafine hydrochloride and tolnaftate were mediated by the stimulation of PDE. AC-inhibiting azole derivatives more effectively reduced cAMP or IL-4 and IL-5 production than PDE-stimulating terbinafine hydrochloride or tolnaftate.

It is reported that the activation of the CD3 molecule leads to the moderate but significant increase of cAMP in T cells (Kvanta *et al*, 1990; Bihoreau *et al*, 1991). Though CD3 does not directly couple to AC, the ligation of CD3 triggers the activation of phospholipase C, and the phospholipase C-mediated signals activate AC (Bihoreau *et al*, 1991; Kvanta *et al*, 1990). The activated phospholipase C generates inositol 1,4,5-triphosphate and diacylglycerol; the former induces the intracellular Ca^{2+} mobilization and the latter activates protein kinase C (Bihoreau *et al*, 1991). It is reported that Ca^{2+} forms a complex with cytosolic calmodulin and the Ca^{2+}/calmodulin complex binds to and activates AC catalytic subunits, whereas protein kinase C activates AC catalysts by phosphorylation (Bihoreau *et al*, 1991; Iyengar, 1993). Protein kinase C-mediated phosphorylation may also downregulate the activity of the inhibitory guanine-nucleotide-binding protein (Gi)-α subunit, which binds to and inactivates AC catalysts (Gordeladze *et al*, 1989; Chen and Iyengar, 1993), and/or promote the activity of the stimulatory guanine-nucleotide-binding protein (Gs)-α subunit, which binds to and activates AC catalysts (Bell *et al*, 1985), and both mechanisms may lead to the activation of AC. As the stimulation of CD28 also triggers the activation of phospholipase C (Nunes *et al*, 1993), the cross-linking of CD3 and CD28 may indirectly activate AC via phospholipase C-mediated signaling pathways in T cells.

In this study, the anti-CD3/CD28-mediated increase of cAMP correlated with the anti-CD3/CD28-induced IL-4 and IL-5 secretion, but did not correlate with that of IFN-γ and IL-2. The PKA inhibitor H-89 reduced the anti-CD3/CD28-induced IL-4 and IL-5 secretion, but not IL-2 and IFN-γ. These indicate that the anti-CD3/CD28-mediated cAMP signal appeared to be required for the Th2 cytokine (IL-4 and IL-5) production while dispensable for that of Th1 cytokines (IFN-γ and IL-2). Previous studies also reported that the cAMP signal stimulated Th2 cytokine production, although some conflicting data are also seen; cAMP-elevating agents, prostaglandin E_2 or cholera toxin, upregulated IL-4 and IL-5 transcription in concanavalin A-primed and ionophore plus phorbol myristate acetate-restimulated murine $CD4^+$ T cells (Lacour *et al*, 1994). Bt_2cAMP activated the IL-5 promoter in murine thymoma EL-4 cells in synergy with phorbol myristate acetate (Lee *et al*, 1993). On the other hand, prostaglandin E_2 did not alter IL-4 production in murine thymoma EL-4 and Th2 cell line D10.G4.1 (Novak and Rothenberg, 1990) or rather reduced concanavalin A-induced IL-4 secretion in human peripheral blood T cells (Borger *et al*, 1996). Thus the regulatory effects of cAMP on Th2 cytokine production may vary depending on the T cell activation status, costimulatory signals, and/or the elevated cAMP level. Our present results indicate that anti-CD3/CD28-induced Th2 cytokine production in human nonprimed T cells may require the transient and moderate rise of cAMP, 2–4-fold of basal level. The promotion of Th2 production by cAMP may possibly be mediated by transcription factor GATA-3, whose binding sites exist on IL-5 and IL-4 promoters and are especially indispensable for IL-5 gene expression (Zhang *et al*, 1997); Bt_2cAMP enhanced DNA-binding activity of GATA-3 in murine Th2 clones (Zhang *et al*, 1997). Alternatively, cAMP may promote IL-4 and/or IL-5 transcription indirectly via another transcription factor CCAAT/enhancer binding protein (C/EBP) whose binding sites exist on human IL-4 and IL-5 promoters (Davydov *et al*, 1995; Cousins *et al*, 2000). It is reported that cAMP induces the synthesis of C/EBPβ by activating cAMP response element binding protein (CREB) via PKA as C/EBPβ promoter contains CREB binding sites (Niehof *et al*, 1997). cAMP also promotes the nuclear translocation of C/EBPβ via PKA-mediated phosphorylation (Metz and Ziff, 1991; Chinery *et al*, 1997). Another possible mechanism is that cAMP/PKA pathway may amplify the autoinduction of IL-4 as IL-4 amplifies IL-4 release from activated $CD4^+$ T cells (Lacour *et al*, 1994).

In contrast, Th1 cytokine production is suppressed by the accumulation of cAMP. cAMP activates PKA and the PKA-phosphorylated CREB and/or activating transcription factor-1 competitively inhibits the binding of activator protein-1 to the IFN-γ promoter and thus suppresses the promoter activity (Penix *et al*, 1996). cAMP-activated PKA may alter the composition of transcription factor complex, nuclear factor of activated T cells, to an unfavorable form for the binding to IL-2 promoter (Lee *et al*, 1993; Tsuruta *et al*, 1995). cAMP also inhibits the binding of nuclear factor κB (p50/p65) heterodimer to IL-2 promoter, which may lead to the suppression of IL-2 transcription (Tsuruta *et al*,

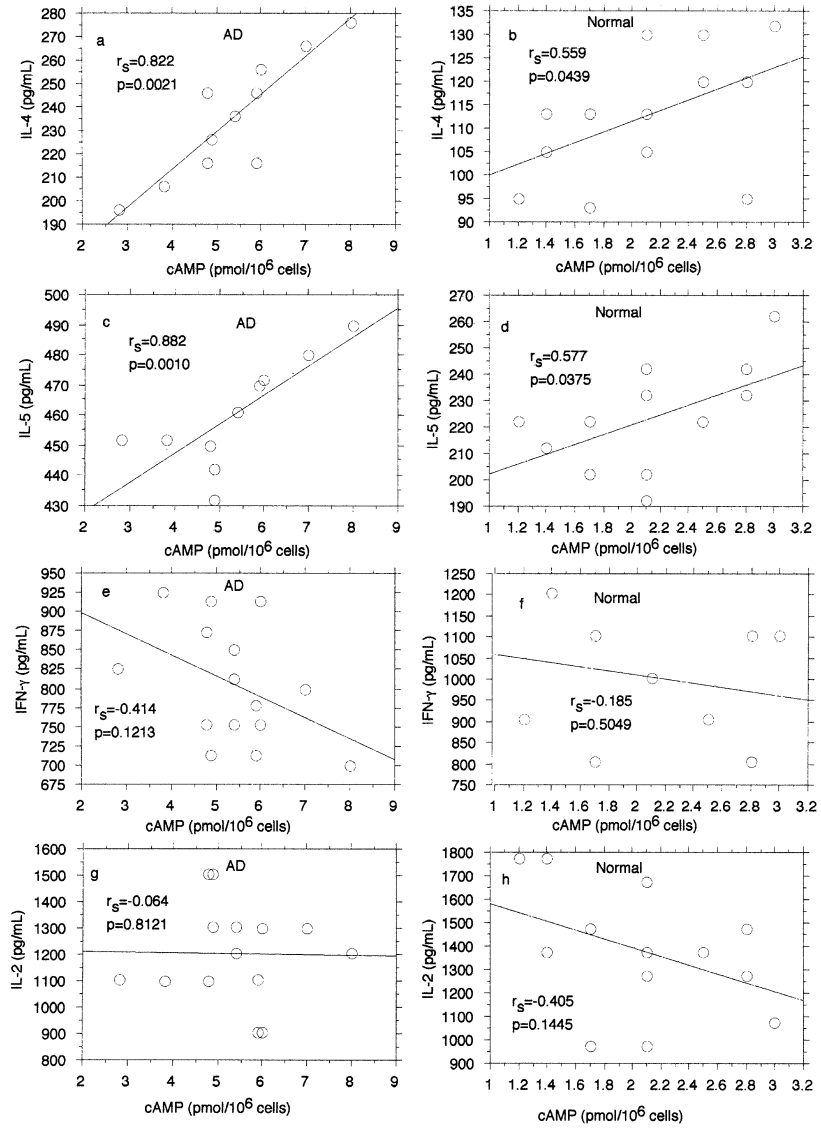

Figure 5. Correlation between anti-CD3/CD28-induced cAMP increase and anti-CD3/CD28-induced IL-4, IL-5, IFN-γ, or IL-2 secretion in AD and normal T cells. (*a*, *b*) IL-4 secretion, (*c*, *d*) IL-5 secretion, (*e*, *f*) IFN-γ secretion, (*g*, *h*) IL-2 secretion. T cells from 15 AD patients and 14 normal donors were incubated in anti-CD3 plus anti-CD28-precoated plates. The intracellular cAMP level was analyzed after 5 min, whereas cytokine secretion was assayed after 48 h, and the background values without anti-CD3/CD28 were subtracted. The anti-CD3/CD28-induced increase of cAMP is shown as the x-axis, whereas anti-CD3/CD28-induced cytokine secretion is shown as the y-axis. The 95% confidence intervals are shown. Background cAMP level and cytokine secretion were described in the footnote for **Table I**.

1995). Thus Th1 cytokine production appears to be suppressed by the excessive and prolonged accumulation of cAMP, e.g., prostaglandin E_2 elevates intracellular cAMP up to 85-fold of basal level (Snijdewint *et al*, 1993). The moderate and transient cAMP increase by anti-CD3/CD28, however (**Fig 4*a***), may not at least suppress Th1 cytokine production as Th1 production was not significantly enhanced by the anti-mycotic-induced reduction of cAMP (**Fig 1*c*, *d***).

The anti-CD3/CD28-induced increase of AC activity and the resultant cAMP signal were greater in AD T cells than in normal T cells (**Fig 4**, **Table III**), and this may contribute to the enhanced IL-4 and IL-5 secretion in the former. This indicates that AC in AD T cells may be more sensitive to anti-CD3/CD28-mediated signals, protein kinase C, and/or Ca^{2+}, compared with that in normal donors' T cells. This may possibly be due to the difference in AC isoform composition between AD and normal T cells. To date, eight different isoforms of AC have been isolated (Iyengar, 1993); protein kinase C stimulates AC types 2 and 7 (Jacobowitz and Iyengar, 1994; Watson *et al*, 1994), and Ca^{2+}/calmodulin stimulates AC types 1 and 3, whereas Ca^{2+} directly inhibits AC types 5 and 6 (Iyengar, 1993). In particular, protein kinase C activates AC type 2 not only by direct phosphorylation of the catalytic subunit but also by suppressing the inhibitory function of Gi-2α on the catalyst (Chen and Iyengar, 1993). AC types 1 or 3 have not been detected in human T cells; however, several reports support that AC in human T cells is activated by Ca^{2+}/calmodulin (Kvanta *et al*, 1990; Bihoreau *et al*, 1991). Thus the amounts of AC types 2 and 7 and/or 1 and 3 may be increased and/or those of AC types 5 and 6 may be reduced in AD T cells as compared with normal T cells. It is also plausible that the protein kinase C isoform(s), which can selectively phosphorylate AC catalysts and/or Gi/Gs, may be more enriched in AD T cells; Gi-2 α is phosphorylated by protein kinase C α and ε but not by δ (Murthy *et al*, 2000), indicating the abundance of protein kinase C α and/or ε in AD T cells. Alternatively, AC catalysts and/or Gi/Gs may be more accessible to the activated protein kinase C in AD T cells; the phosphorylation sites of AC catalysts and/or Gi/Gs may be more exposed in AD T cells and/or AD T cells may more abundantly contain certain anchoring protein(s) termed receptor(s) for activated C kinase, which may bind to and target the activated protein kinase C preferentially to the phosphorylation sites on AC catalysts and/or Gi/Gs (Mochly Rosen and Gordon, 1998). The putative promotion of the cross-talk between Ca^{2+}/protein kinase C signals and AC may contribute to the enhanced Th2 cytokine production in AD T cells (**Table I**), and thus the precise mechanism(s) for the promotion should be elucidated in further

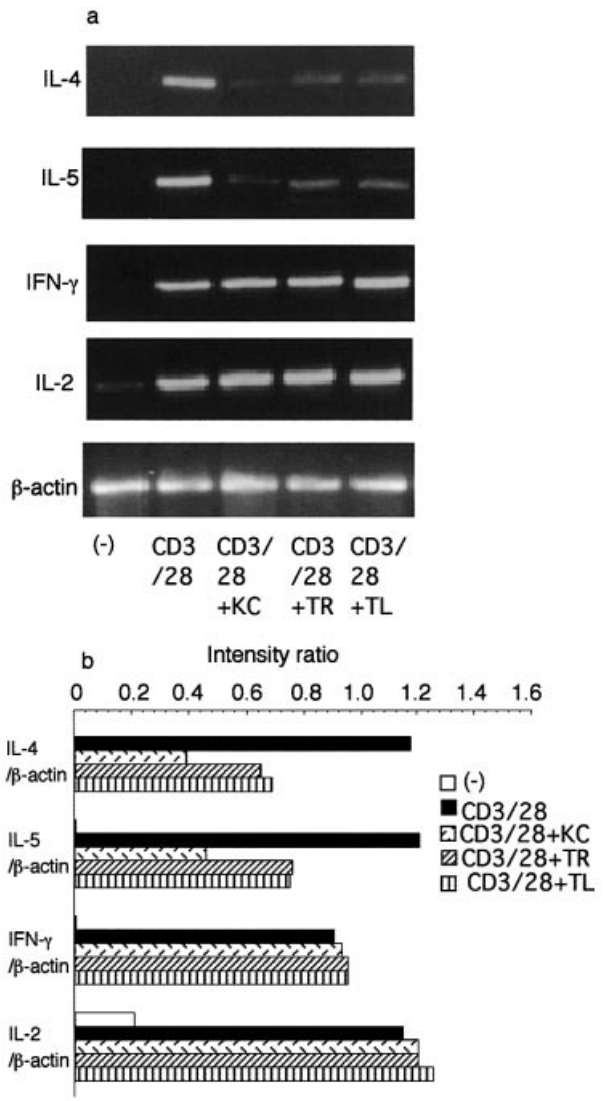

Figure 6. The effects of anti-mycotics on anti-CD3/CD28-induced mRNA expression for Th2 and Th1 cytokines. T cells from an AD patient were preincubated for 30 min with medium alone or with medium containing ketoconazole, terbinafine hydrochloride, or tolnaftate (each 1 μM), then seeded to anti-CD3 plus anti-CD28-precoated plates, and incubated in the presence or absence of respective anti-mycotics for another 6 h. RNA was extracted and reverse transcription–PCR products were analyzed by electrophoresis (*a*) and the intensity of the products was determined by densitometry (*b*). The data represent five separate experiments using T cells from five different AD patients.

studies. Another possible mechanism is that anti-CD3/CD28 may induce, to a greater extent, secondary signals, protein kinase C activation, and/or intracellular Ca^{2+} signal in AD T cells than in normal T cells, which may lead to greater AC activation in the former. This possibility is, however, rather unlikely as IL-2 and IFN-γ production (which is more dependent on protein kinase C than Th2 cytokine production) was not increased in AD T cells compared with that in normal T cells.

Previous studies reported that azole derivatives reduced the activity of AC; azole derivatives ketoconazole, itraconazole, and miconazole inhibited basal and corticotropin-releasing hormone, cholera toxin, NaF, or forskolin-stimulated AC activity in rat anterior pituitary cells (Stalla *et al*, 1988, 1989). These reports suggest that the azole derivatives may directly inhibit the activity of AC catalytic subunit(s) and favor our present results. Azole derivatives also inhibit membrane-associated AC activity of *Candida albicans* by changing the conformation of the AC-surrounding membrane microenvironment, such as membrane fluidity (Surarit and Shepherd, 1987). Opposing results were also reported, however; ketoconazole alone and in synergy with thyroid-stimulating hormone increased cAMP release from rat thyroid cells, indicating the ketoconazole-induced stimulation of AC (Kohan *et al*, 1992). Thus the effects of azole derivatives on AC appeared to vary depending on the cell types. This is possibly because the composition of AC isoforms differs with cell type and azole derivatives may differentially inhibit some isoform(s) while stimulating the other isoform(s). Thus the azole-inhibitable AC isoform(s) should further be elucidated.

Azole derivatives did not alter the activity of PDE from human T cells in this study. Discrepant results are, however, reported; azole derivatives inhibited the activity of Ca^{2+}/calmodulin-dependent PDE (PDE type 1) derived from beef heart by interacting with calmodulin (Hegemann *et al*, 1993). The discrepancy from our present results may be due to the difference in PDE isoforms depending on the cell and tissue types; the major PDE types in normal human T cells are PDE3, 4, and 7, whereas PDE1 activity is very low (Tenor *et al*, 1995; Giembycz *et al*, 1996; Erdogan and Houslay, 1997; Li *et al*, 1999b). Hegemann *et al* (1993) also reported that azole derivatives inhibited the activity of calmodulin-independent (non-PDE1) PDE activity from the beef heart; however, this effect only occurs at extremely higher concentrations, e.g., IC_{50} 262.5 μM for ketoconazole, and may not occur in the concentration range (= 10 μM) used in our study.

In this study, PDE activity is slightly increased after anti-CD3/CD28 stimulus, which may be due to the PKA activation by anti-CD3/CD28-induced cAMP; it is reported that PKA phosphorylates PDE4D3, which induces the rapid and reversible activation of PDE4D3 (Sette and Conti, 1996). Terbinafine hydrochloride and tolnaftate further increased the PDE activity in anti-CD3/CD28-stimulated T cells, which resulted in the suppression of cAMP signal, although their cAMP-reducing effects were lower than those of AC-inhibiting azole derivatives. There were few reports regarding the effects of terbinafine hydrochloride or tolnaftate on PDE activity; treatment with tolnaftate did not alter PDE activity of *Microsporum gypseum* cells (Gupta *et al*, 1991). Tolnaftate did not alter the activity of PDE1 from beef heart (Hegemann *et al*, 1993); however, the effects of tolnaftate or terbinafine hydrochloride on PDE or AC in human T cells have not been precisely examined. We are now studying which PDE isoform(s) may be stimulated by these drugs.

The activation of T cells with anti-CD3/CD28 on T cells reflects the interaction with antigen-presenting cells, as antigen-bound major histocompatibility complex molecules and B7 molecules on antigen-presenting cells interact with T cell receptor/CD3 complexes and CD28 on T cells, respectively. Thus the anti-mycotics may also suppress the Th2 cytokine production of T cells, which interact with the cells presenting allergens such as house dust mite, and this possibility is now under investigation. In this study, azole and nonazole anti-mycotics inhibited Th2 cytokine production at 0.01–1 μM with an optimal concentration of 1 μM, which is close to the concentrations obtained by the oral administration of these drugs; the peak serum concentration was 0.71–0.85 μM for itraconazole (Warnock, 1989). It is thus indicated that these anti-mycotics may suppress Th2 cytokine production *in vivo*. It has been recently reported that Th2 cytokines, IL-4 and IL-5, drive the initial phase of AD, and attract and activate macrophages or eosinophils in the skin lesions (Grewe *et al*, 1998), whereas in a later phase IFN-γ-producing T cells predominantly infiltrate and continue the inflammatory responses (Thepen *et al*, 1996; Grewe *et al*, 1998). Thus the anti-mycotics may prevent the initiation of atopic eczema rather than suppress the ongoing skin inflammation. As patch tests with aeroallergens such as house dust mites trigger Th2-skiewed responses in the skin of AD patients (Sager *et al*, 1992), we should further examine if anti-mycotics may inhibit the patch test reaction

to such aeroallergens in AD patients. A previous study reported that topical treatment with ketoconazole suppressed erythematous inflammatory skin reaction to heat-killed *Staphylococcus aureus* in guinea-pigs (van Custem *et al*, 1991); however, there have been no studies regarding the effects of anti-mycotics on patch test reactions to aeroallergens. We should also examine if the anti-mycotics may *in vivo* suppress Th2 cytokine production in AD patients by comparing the Th1/Th2 cytokine profiles between premedication and postmedication and should also assess if these agents might prevent the relapse of atopic eczema.

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